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Regular paper

Fluorescence of the tri-cyclic adenine and isoguanine derivatives and their ribosides: possible analytical applications*

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Fluorescent tri-cyclic purine analogs, derivatives of isoguanine and adenine, were examined as potential substrates of purine-nucleoside phosphorylase. It was found previously that etheno- derivatives of both compounds are ribosylated in phosphate-free media, but ribosylation places in some instances differ from purine N9. New ribosides are examined as potential substrates of human blood PNP and indicators of this enzyme. Of these, N⁶-riboside of 1,N⁶-etheno-adenine was found the most promising.

Key words: nucleobase/nucleoside analogs; fluorescence; purine nucleoside phosphorylase; enzyme activity, blood lysates

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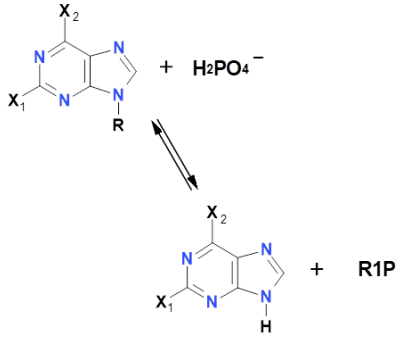
Abbreviations: εAde, ethenoadenine, 1,N⁶-ethenoadenine (imidazo[2,1-i]purine); εAdo, etheno-adenosine, 1,N⁶-ethenoadenosine; isoGua, isoguanine, 2-oxoadenine; isoGuo, isoguanosine, 2-oxoadenosine; εisoGua, 1,N⁶-etheno-isoguanine (IUPAC name: 5-oxo-imidazo[1,2-i]purine); PNP, purine-nucleoside phosphorylase; EDTA, ethylenediamine-tetraacetic acid

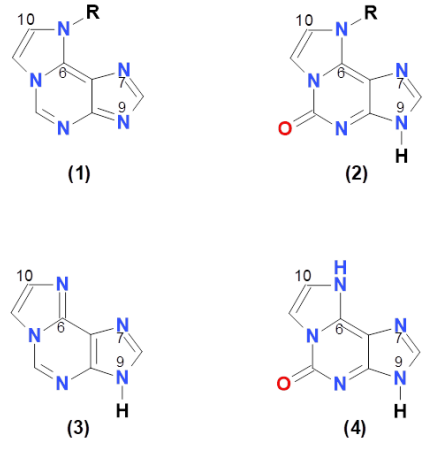
INTRODUCTION

Tri-cyclic analogs of the canonical purines are frequently used as fluorescent probes in enzymological research. The best known example is 1,N⁶-etheno-adenosine (εAdo) and analogs (Leonard, 1984; Leonard, 1985), but other fluorescent derivatives are also known (Virta *et al.*, 2004; Wang *et al.*, 2017). Tri-cyclic analogs and their ribosides are characterized by moderate biological activity, but some of them reveal promising anti-viral properties (Janz-Wechmann *et al.*, 2015). They are known to react with many enzymes of purine metabolism (Leonard, 1984), and are important intermediates in the process of chemical mutagenesis induced by vinyl chloride (Singer & Kuśmierek, 1982; Chatterjee & Walker, 2017).

In our laboratory we are working on an enzyme purine-nucleoside phosphorylase (PNP, E.C. 2.4.2.1, see Scheme 1), responsible for the regulation of the nucleoside concentrations within the living cells, and a target of many types of pharmaceutical interventions, including gene therapy of the inherited immunological disorders (Bzowska *et al.*, 2000; Grunebaum *et al.*, 2013). We have shown that PNP isolated from *E. coli*, which is known to possess a broad specificity toward various base and nucleoside analogs (Bzowska *et al.*, 2000; Yehia *et al.*, 2017) is also active towards tri-cyclic εAdo and its 2-aza analog (Wierzchowski *et al.*, 2017). We have found also that 1,N⁶-etheno-adenine (εAde), 1,N²-etheno-guanine and 1,N⁶-etheno-isoguanine (εisoGua) are good substrates for PNP from *E. coli* (Fig. 2, left) in the reverse (synthetic) pathway, with catalytic and Michaelis' constants sometimes comparable to those obtained for the enzymatic ribosylation of the parent guanine (Stachelska-Wierzchowska *et al.*, 2018; Stachelska-Wierzchowska *et al.*, 2019). The ribosides obtained in these reactions are



Scheme 1. General course of the reaction catalyzed by purine-nucleoside phosphorylase (R, β-D-ribose; R1P, α-D-ribose-1-phosphate).



Scheme 2. Structure of artificial PNP substrates examined in this work (R, ribose).
(**1**) N⁶-β-D-ribosyl-1,N⁶-ethenoadenine, (**2**) N⁶-β-D-ribosyl-1,N⁶-ethenoisoguanine, and products of their phosphorolysis: (**3**) 1,N⁶-ethenoadenine and (**4**) 1,N⁶-ethenoisoguanine. Note that the purine numbering is applied.

not necessarily identical with those produced chemically, with ribosylation sites varying for different PNP forms.

The purpose of the present paper is examination of substrate properties of new fluorescent ribosides toward the human blood enzyme, and their analytical potential in clinical investigations. We have previously identified two such substrates, isomeric ribosides of 2,6-diamino-8-azapurine (Wierzchowski *et al.*, 2014), and now extend our work to include tri-cyclic ribosides with different spectral characteristics.

Human PNP, as well as other mammalian forms of this enzyme, belongs to the second (trimeric) class within the broad family of PNP (Bzowska *et al.*, 2000), and its substrate specificity is different than that of the hexameric (bacterial) forms. In particular, the trimeric forms of PNP are inactive towards adenosine and some derivatives (Bzowska *et al.*, 2000; Yehia *et al.*, 2017). We therefore considered only those ribosides, which were previously shown to be substrates for the calf enzyme (Stachelska-Wierzchowska *et al.*, 2018, 2019). We have chosen some non-canonical ribosides of etheno-adenine and etheno-isoguanosine (Scheme 2).

MATERIALS AND METHODS

Synthesis of the 1,N^6-etheno-isoguanine (base, **4**) from isoguanine and chloroacetaldehyde has been described previously (Stachelska-Wierzchowska *et al.*, 2019). This reaction is slow (ca. 7 days at room temperature) but the reaction product crystallized easily from neutralized medium and the reaction yield (~70%) was sufficient. Chemo-enzymatic syntheses and identification of two described ribosides of 1,N^6-etheno-adenine and 1,N^6-etheno-isoguanine (**1** and **2**) were described elsewhere (Stachelska-Wierzchowska *et al.*, 2018; Stachelska-Wierzchowska *et al.*, 2019). These compounds were stored as frozen solutions. Recombinant calf spleen PNP was obtained from Prof. Agnieszka Bzowska (Warsaw University, Poland).

Blood samples were obtained as leftovers from glucometric measurements of one of the authors (J.W.). 10 μL blood samples were lysed in 0.5 mL of 2 mM phosphate buffer, pH 7, containing 0.5 mM EDTA and an aliquot (~0.2 mM) of dithiothreitol. The hemolysates were kept at 5°C.

Fluorescence spectra were measured on a Varian Eclipse instrument (Varian Corp., Palo Alto, CA, USA). Spectral resolution was typically 5 nm (emission path) and 2.5 nm (excitation path). UV absorption and kinetic experiments were performed on a Cary 5000 (Varian) thermostated spectrophotometer. Enzymatic reactions were carried out at 25°C. Fluorescence yields were determined relative to tryptophan (0.15) or 1,N^6-ethenoadenosine in water (0.56; Leonard, 1984). Spectra were measured in semi-micro 1 mL cuvettes, pathlength 4 mm, to diminish the inner-filter effect and hemoglobin absorption. Substrate/product concentrations were calculated using known molar extinction coefficients: 8200 $M^{-1}cm^{-1}$ for etheno-adenine (274 nm) and 7000 $M^{-1}cm^{-1}$ for etheno-isoguanine at 291 nm (Stachelska-Wierzchowska *et al.*, 2018; Stachelska-Wierzchowska *et al.*, 2019). Typically, substrate concentrations in fluorescence measurements were 5-fold lower than those measured by UV absorption.

RESULTS AND DISCUSSION

It has been shown previously, that enzymatic ribosylation of some nucleobase analogs with PNP as a biocatalyst leads to non-typical ribosides, with ribose moiety attached not necessarily to purine N9, but also to other nitrogen atoms (Stachelska-Wierzchowska *et al.*, 2013; Stachelska-Wierzchowska *et al.*, 2016; Stachelska-Wierzchowska *et al.*, 2018; Stachelska-Wierzchowska *et al.*, 2019). In particular, while the *E. coli* PNP directs the ribosyl group predominantly to the N9 of etheno-adenine, the calf enzyme leads to almost exclusively N^6-riboside (Stachelska-Wierzchowska *et al.*, 2018). In 1,N^6-ethenoisoguanine, the situation is even more complex, since typically mixtures of various ribosides are produced (Stachelska-Wierzchowska *et al.*, 2019).

Spectral properties of new ribosides were presented in previous papers (Stachelska-Wierzchowska *et al.*, 2018; Stachelska-Wierzchowska *et al.*, 2019) and those of three 1,N^6-ethenoisoguanine ribosides are summarized in Fig. 1.

The most interesting, from analytical point of view, were those ribosides which were generated by calf spleen PNP, that is, N^6-β-D-ribosides. We have shown that these ribosides (see Scheme II, above) were also excellent substrates for the *E. coli* PNP, and phosphorolytic

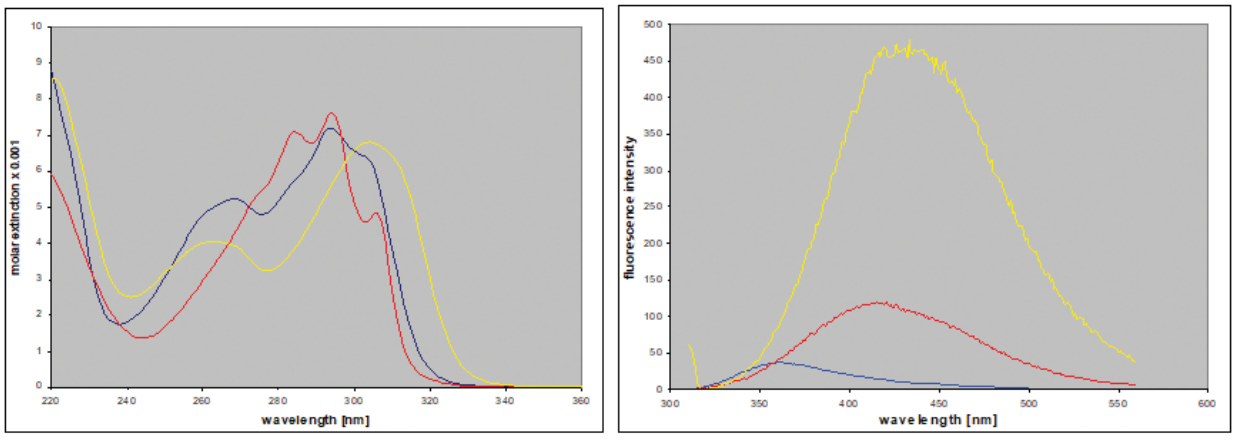


Figure 1. Electronic absorption (left) and fluorescence (right) spectra of three ribosides of 1,N^6-etheno-isoguanine, purified by HPLC. Spectra of N9- (red color) and N7-β-D-ribosides (blue) were measured in 50 mM phosphate buffer, pH 6.5, and that of N^6-riboside (**2**, yellow), at pH 7. The respective quantum yields are 0.34, 0.04 and 0.66. Data from Stachelska-Wierzchowska *et al.* (Stachelska-Wierzchowska *et al.*, 2019).

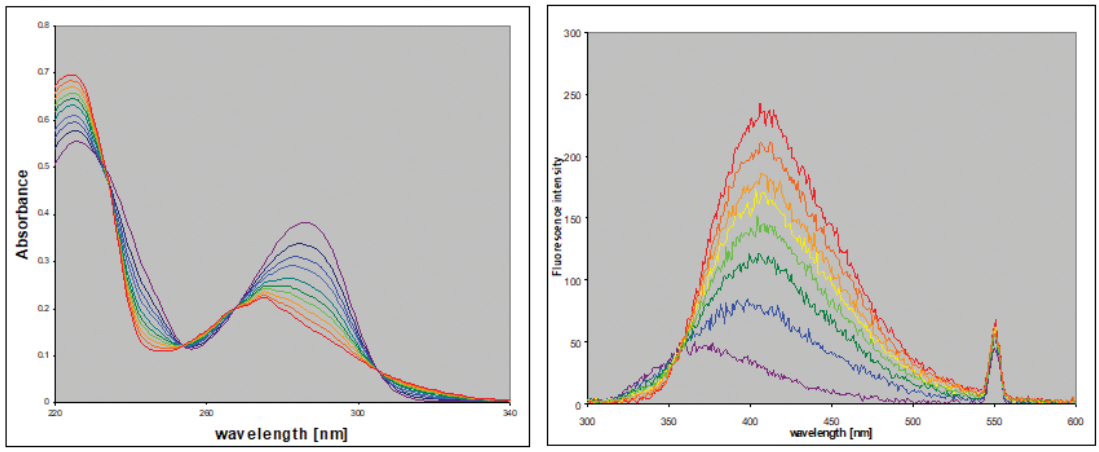


Figure 2. Spectral (left) and fluorescence (right) changes observed during the phosphorolysis of N⁶-β-D-ribosyl-1,N⁶-etheno-adenine (1) with *E. coli* PNP as a catalyst, in the phosphate buffer, pH 7, at 25°C.
Initial substrate concentration in UV experiment was 66 μM, and for emission 13.2 μM. Cuvettes of a reduced pathlength (4 mm) were used. Fluorescence excitation was at 275 nm. Time intervals: 5 min for UV absorption, 10 min for fluorescence. Final curves are drawn in red.

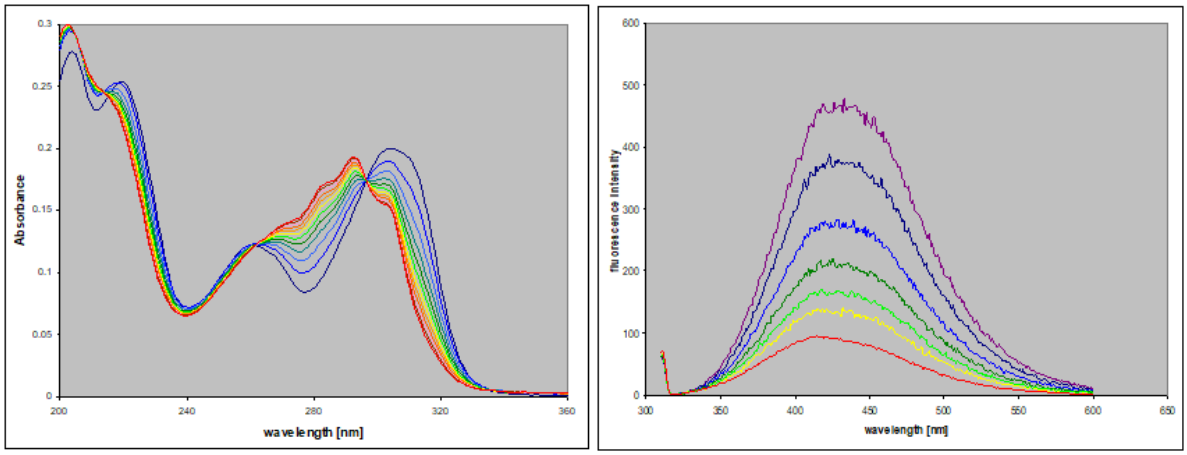


Figure 3. Spectral (left) and fluorescence (right) changes observed during the phosphorolysis of N⁶-β-D-ribosyl-1,N⁶-etheno-isoguanine (2) in the phosphate buffer, pH 7.
Initial substrate concentrations: 68 μM for UV, 14 μM for emission. Fluorescence was excited at 310 nm. Time intervals are as in Fig. 2. Final curves are drawn in red.

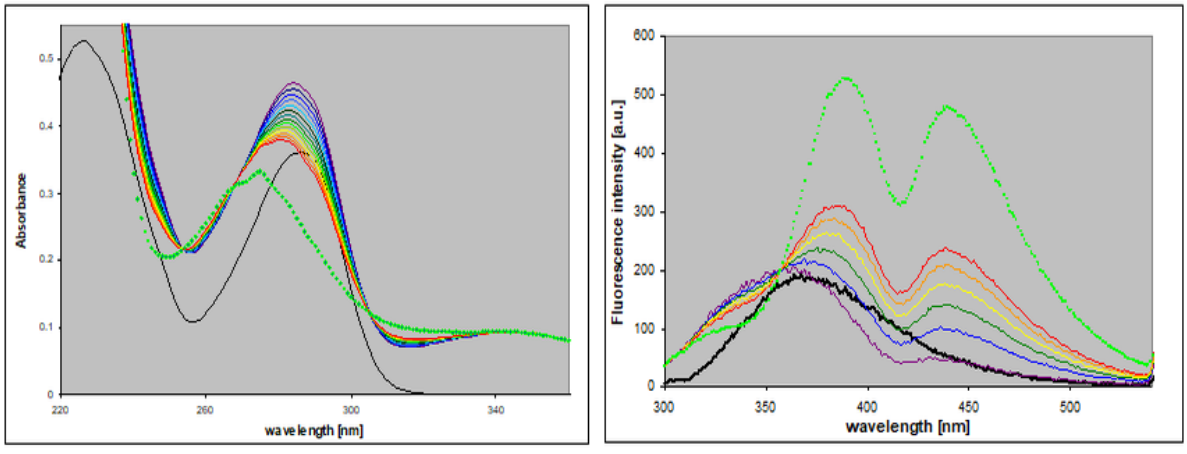


Figure 4. Spectral (left) and fluorescence (right) changes observed during the phosphorolysis of N⁶-β-D-ribosyl-1,N⁶-etheno-adenine (1) with *blood lysate* as a catalyst, in the phosphate buffer, pH 7, at 25°C.
Experimental conditions were exactly as in Fig. 2, above. Final curves (points) were obtained by spiking blood sample with the purified *E. coli* PNP. Black curves refer to purified substrate (**1**) spectra. Time intervals were 5 minutes for UV absorption, 10 minutes for fluorescence. Fluorescence minimum at 410 nm is due to light re-absorption by hemoglobin (Slater band). Note that the isosbestic and isoemissive points are strictly maintained.

reactions were easily followed by UV absorption of fluorescence spectroscopy (Figs. 2 and 3). The new ribosides are fairly stable, and could be stored for months in stock solutions at –5°C (not shown).

Human blood is particularly rich in PNP activity, which is located mainly in erythrocytes (Bzowska *et al.*, 2000). We have shown previously that some phosphorolytic reactions are easily observed spectrally or fluorimetrically using 1000-fold diluted whole blood lysates as a catalyst in ca. 50 mM phosphate buffer (Wierzchowski *et al.*, 2002; Wierzchowski *et al.*, 2014). We have therefore examined the new ribosides as potential substrates for the human PNP, using the same methodology (except optical pathlength of the cuvettes, reduced now to 4 mm).

Experiments with 1000-fold diluted blood lysates has shown that only one of the examined substrates, of N^6-β-D-ribosyl-1,N^6-etheno-adenine (**1**), was readily phosphorolysed (Fig. 4) with calculated reaction rate of ~0.45 μM/min at substrate concentration 66 μM. Phosphorolysis was easily observed spectrophotometrically as well as fluorimetrically. Hemolysate optical background does not interfere with the measurements, except visible re-absorption near the Slater band of hemoglobin at 410 nm (Fig. 4). Blood proteins contribute somewhat to the overall fluorescence with excitation at 275 nm, but with 1000-fold sample dilution this fluorescence, visible as short-wavelength inflection on Fig. 4, right panel, is low and negligible at λ ~ 430 nm, where the measurements are the most accurate.

The second substrate, the highly fluorescent N^6-β-D-ribosyl-1,N^6-etheno-isoguanine (**2**), was apparently inactive (rate <0.01 μM/min) with the same blood sample, although it was rapidly phosphorolysed by the purified *E. coli* PNP (Fig. 3) as well as by the calf enzyme. This result was somewhat surprising, since the human enzyme belongs to the same class of trimeric PNP, and shows homology of >70% with calf PNP (Bzowska *et al.*, 2000). At present, we are unable to explain this phenomenon.

The experimental conditions of the presented reactions need to be optimized, for applications to clinical analyses. This refers particularly to buffer pH, substrate concentration (the apparent K_m for the human enzyme) and excitation wavelength. With conditions fulfilled, the proposed assay will be probably much more sensitive than those described previously.

CONCLUDING REMARKS

We have described two novel, both fluorescent and fluorogenic, substrates for PNP. One of these, N^6-β-D-ribosyl-1,N^6-etheno-adenine (**1**), can be used to quantitate PNP activity in human blood. Possible applications include early detection of immunological deficiencies (Grunebaum *et al.*, 2013). The second substrate, N^6-β-D-ribosyl-1,N^6-etheno-isoguanine (**2**), can be used to selectively detect bacterial PNP activity in biological samples, with possible use in the investigations of the suicidal gene therapy of cancer, utilizing bacterial PNP to generate *in situ* cytotoxic nucleobase analogs (Karjoo *et al.*, 2017).

Various assays, including fluorimetric, were previously proposed for this enzyme (Bzowska *et al.*, 2000; Wierzchowski *et al.*, 2002; Wierzchowski *et al.*, 2014), but their sensitivity was not always satisfactory, mostly because of slow reaction rates of the artificial substrates. Therefore search for new, more sensitive substrates is continued.

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